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Potential mechanisms of marked hyperoxaluria not due to primary hyperoxaluria I or II

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Background. Hyperoxaluria may be idiopathic, secondary, or due to primary hyperoxaluria (PH). Hepatic alanine:glyoxylate aminotransferase (AGT) or glyoxylate/hydroxypyruvate reductase (GR/HPR) deficiency causes PHI or PHII, respectively. Hepatic glycolate oxidase (GO) is a candidate enzyme for a third form of inherited hyperoxaluria.

Methods. Six children were identified with marked hyperoxaluria, urolithiasis, and normal hepatic AGT ($N = 5$) and GR/HPR ($N = 4$). HPR was below normal and GR not measured in one. Of an affected sibling pair, only one underwent biopsy. GO mutation screening was performed, and dietary oxalate ($Diet_{ox}$), enteric oxalate absorption (EOA) measured using [$^{13}C_2$] oxalate, renal clearance (GFR), fractional oxalate excretion (FE_{ox}) in the children, and urine oxalate in first-degree relatives (FDR) to understand the etiology of the hyperoxaluria.

Results. Mean presenting age was 19.2 months and urine oxalate 1.3 ± 0.5 mmol/1.73 m^2/24 h (mean ± SD). Two GO sequence changes (T754C, IVS3 – 49 C>G) were detected which were not linked to the hyperoxaluria. $Diet_{ox}$ was 42 ± 31 mg/day. EOA was 9.4 ± 3.6%, compared with 7.6 ± 1.2% in age-matched controls ($P = 0.33$). GFR was 90 ± 19 mL/min/1.73 m^2 and FE_{ox} 4.2 ± 1.4. Aside from the two brothers, hyperoxaluria was not found in FDR.

Conclusions. These patients illustrate a novel form of hyperoxaluria and urolithiasis, without excess $Diet_{ox}$, enteric hyperabsorption, or hepatic AGT, GR/HPR deficiency. Alterations in pathways of oxalate synthesis, in liver or kidney, or in renal tubular oxalate handling are possible explanations. The affected sibling pair suggests an inherited basis.

Childhood urolithiasis in the United States is estimated to account for between 1 in 1000 to 1 in 7600 hospital admissions [1]. Metabolic risk factors account for greater than 50% of cases [2–4]. Idiopathic hypercalciuria is identified in up to one half of patients with a recognized metabolic risk factor, and the majority of stones are comprised of calcium oxalate or calcium phosphate [2–5]. Hyperoxaluria in pediatric stone disease is less frequent, observed in 1 to 20% of children who undergo metabolic evaluation for urolithiasis [3, 4, 6], and may be primary, secondary or idiopathic. Marked and persistent hyperoxaluria, exceeding 1.0 to 1.5 mmol/1.73 m^2/24 h, most often heralds one of the inherited hyperoxalurias due to hepatic enzyme deficiency of alanine: glyoxylate aminotransferase (AGT) in type I primary hyperoxaluria (PHI) or glyoxylate/hydroxypyruvate reductase (GR/HPR) in type II primary hyperoxaluria (PHII). Glycolate oxidase (GO), another hepatic enzyme contributing to glyoxylate synthesis, a key precursor step of endogenous oxalate, is a candidate enzyme for a third form of inherited hyperoxaluria (Fig. 1). Williams, Cregeen and Rumsby recently isolated and expressed the cDNA for human liver GO [7].

Enteric hyperoxaluria is typically evident from a clinical history or findings of intestinal malabsorption, is moderate to marked, and varies widely with dietary changes (0.7 to >1.0 mmol/1.73 m^2/24 h) [8]. Other secondary forms of hyperoxaluria include dietary oxalate excess and increased gastrointestinal absorption not due to gastrointestinal disease or resection. Recent evidence suggests a role for oxalate-degrading gut flora in the latter form of hyperoxaluria [9]. Pyridoxine is an essential cofactor of AGT and its deficiency is a theoretical consideration—though to date unproven—as a clinical cause of urolithiasis in humans. Idiopathic hyperoxaluria, a diagnosis of exclusion, is generally mild (<0.7 mmol/1.73 m^2/24 h).

Our current study describes six children, from five unrelated families, with moderate to marked hyperoxaluria and calcium oxalate urolithiasis in the absence of any known primary or secondary cause. A novel enzymatic defect of glyoxylate metabolism or oxalate synthesis, either in hepatic or renal cells, or a renal tubular defect of oxalate transport is suggested by the data.

Key words: enteric oxalate absorption, calcium oxalate urolithiasis, alanine:glyoxylate aminotransferase, stone formation, kidney stones, urolithiasis.

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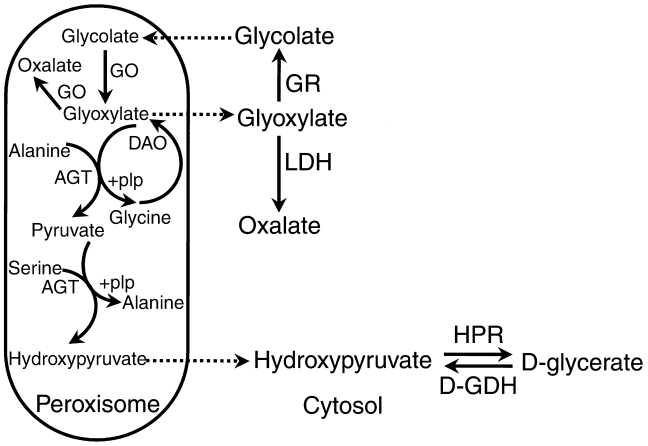

Fig. 1. Schematic representation of the proposed pathways of glyoxylate metabolism in the human liver cell. Abbreviations are: AGT, alanine:glyoxylate aminotransferase; DAO, D-amino acid oxidase; D-GDH, D-glycerate dehydrogenase; GO, glycolate oxidase; GR, glyoxylate reductase; HPR, hydroxypyruvate reductase; LDH, lactate dehydrogenase; plp, pyridoxal phosphate, an essential cofactor of AGT.

METHODS

From 1984 to 2000, six children were identified from five unrelated families who had persistent moderate to marked hyperoxaluria (greater than 0.9 mmol/1.73 m^2/24 h) and calcium oxalate urolithiasis. Percutaneous hepatic biopsies were obtained in five of the children. In the family with two affected siblings, only one child underwent biopsy. Hepatic enzyme analyses for AGT and GR/HPR were performed in the laboratory of Dr. Gill Rumsby using previously published methods [10, 11].

The children were evaluated for secondary causes of hyperoxaluria, including assessment of dietary oxalate, measurement of enteric oxalate absorption using [$^{13}C_2$] oxalate, serum levels of pyridoxine and its metabolites, plasma oxalate concentrations, glomerular filtration rate (GFR), and calculation of renal fractional oxalate excretion. Six age-matched healthy children participated in the [$^{13}C_2$] oxalate absorption study.

First-degree family members completed a 24-hour urine collection for calcium, oxalate, and creatinine. Blood samples for DNA analyses were obtained from the six probands and their first-degree relatives for mutation screening of the *GO* gene. The study protocol was approved by the Mayo Clinic Institutional Review Board (IRB).

Methods for laboratory evaluation

Plasma and urine oxalate were measured by the immobilized oxidase method [12]. Our normal reference range for plasma oxalate is 0.4 to 3.0 μmol/L and for 24-hour urine oxalate 0.11 to 0.46 mmol. When measured values for urine oxalate are corrected for body surface area, the adult normal reference range is applicable in children [13]. All urine samples for oxalate determination were acidified to pH <2 with hydrochloric acid. Urine glycolate and L-glycerate were measured by gas chromatography on two-hour fasting urine specimens [14]. In our laboratory, the normal reference ranges are <70 μg/mg creatinine and <19 μg/mg creatinine, respectively.

Measurements of creatinine, phosphorous, calcium, and uric acid in serum and urine were by standard laboratory methods. Measurement of urine citrate was by the colorimetric method of Natelson, Pincus and Lugovoy [15]. The renal threshold phosphate concentration ($TmPO_4$/GFR) was derived from the nomogram of Walton and Bijvoet using the plasma phosphate concentration and the measured fractional tubular reabsorption of phosphate (TRP) [16]. Age-appropriate pediatric reference ranges for $TmPO_4$/GFR and TRP were obtained from published reports [17, 18]. Fasting urine specimens were screened for organic acids by capillary gas chromatography mass spectrometry [19] and for amino acids by ion exchange chromatography [20].

Renal function was measured by two-hour clearance of non-radiolabeled iothalamate using capillary electrophoresis [21]. Fractional oxalate excretion (FE_{ox}) was calculated from the renal clearance of oxalate and the simultaneous renal clearance of creatinine (C_{Cr}). Values less than unity were considered to represent net renal tubular oxalate reabsorption and values exceeding unity to suggest net renal tubular oxalate secretion [22]. The fractional excretion of uric acid (FE_{UA}) was calculated from simultaneous fasting blood and urine samples of uric acid and creatinine. The FE_{UA} in healthy children decreases as they get older [23–25].

High-pressure liquid chromatography (HPLC) and a radioreceptor assay were used to measure 25-hydroxy and 1,25-dihydroxy vitamin D levels [26, 27]. In children, the circulating concentrations of these vitamin D metabolites vary with age [28]. Blood levels of pyridoxine and its metabolites were also measured using HPLC [29]. Levels of parathyroid hormone were determined by the immunochemiluminometric parathyroid hormone assay (ICMA-PTH) [30]. The normal reference range in our laboratory is 1.0 to 5.2 pmol/L. Stool samples were analyzed for fat using the gravimetric method of Van de Kamer et al [31] and published pediatric reference ranges [32]. Stone recovery was by urologic procedure and stone analysis was by infrared spectroscopy.

Dye-binding assays were used for urine protein quantification, Coomassie Blue for 24-hour collections, and Pyrogallol Red for random samples [33]. Urine osmolality measurement for calculation of urine protein/osmolality ratios (mg/L/mOsm/kg) was by freezing point depression and random urine glucose concentration by the glucose oxidase method (normal ≤25 mg/dL). The normal value for a random urine protein/osmolality ratio is

Table 1. Eight exons of the *GO* gene

Exon/ primer	Sequence (5′ to 3′)	Product length *bp*	Exon/ primer	Sequence (5′ to 3′)	Product length *bp*
1F	GTAGAAAGCAATACATTAAAA		5F	AAGCTGCCTGTTAAGTT	
1R	TTTGTGTAATTTTAAAACATG	249	5R	GAGGAGGAAGACATAGAGATA	226
2F	CTGAAACTCTAAAGCCTTTTA		6F	GAGTCACATTATTGAACTTT	
2R	GGTCGATAAACGTTAGCCTCC	247	6R	TTGTTTTACTGTCAAGTTGTC	243
3F	GGCCCATCTTGATCATC		7F	AAACAAATGAATAAAACAAGT	
3R	TTCCCAGTCAAGATCCCTTTC	342	7R	CCAAGGACCTTGACTTAAA	200
4F	AATGAGCAGTGAACAGCCAA		8F	GGGAAAACGATTACCTG	
4R	CAGAGAATTGAGAGTTGGAATGTC	426	8R	TCTTTGTCAAGTAATACATGC	200
6.5F	AATTGTGGAGGCTGTGGAAG		6.5R	CCCACACTCTTCCCTTTCAA	424

<0.12 in adults [34] and the 95th percentile for normal, non-proteinuric children is 0.23 [35].

Methods for measurement of enteric oxalate absorption

Enteric oxalate absorption was measured in the General Clinical Research Center using a labeled stable isotope of oxalate ($[^{13}C_2]$ oxalate) as described by Liebman and Chai [36]. Except for water, patients and the six age-matched healthy control children were asked to remain fasting after midnight. On the morning of the study, a baseline two-hour urine collection was completed. Patients then ingested a small amount of grape-flavored jello, containing 20 mg/1.73 m^2 of $[^{13}C_2]$ oxalic acid and 100 mg/1.73 m^2 of $[^{12}C_2]$ oxalic acid. No other food was ingested. Three sequential two-hour urine collections followed for the next six hours, during which time only distilled water was taken orally.

Oxalic acid was isolated from 200 μL of acidified urine by applying the sample over a column containing a 1 mL bed volume of Dowex AG 50W-X8 cation exchange resin. The column was then rinsed with 2 × 1 mL 0.1 N HCl and the eluant collected and combined. After drying in a Savant centrifugal evaporator, the sample was derivatized with N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide + 1% t-butyl-dimethylchlorosilane (MTBSTFA + 1% t-BDMCS) in acetonitrile at 70°C for 30 minutes. The oxalate derivative was analyzed on a Hewlett Packard 5989B Gas Chromatograph Mass Spectrometer (Palo Alto, CA, USA) under electron ionization conditions using a DB5MS 30 m × 0.25 mm I.D. × 0.5 μm capillary column (J & W Scientific, a division of Agilent Technologies, Palo Alto, CA, USA). Fragment ions at m/z 261 and m/z 263 were monitored to determine the $[^{13}C_2]$ oxalic acid enrichment. Concentration of oxalate in the urine was measured on a further aliquot by adding $[^{13}C_2]$ oxalic acid as an internal standard and monitoring the same fragment ions. Corrections were applied to account for the levels of $[^{13}C_2]$ oxalate present from the oral dose.

The total amount of $[^{13}C_2]$ oxalic acid (mg) excreted over the six-hour interval after ingestion of the isotope was calculated from the measured concentrations and urine volumes for each of the three two-hour periods. The percent recovery of $[^{13}C_2]$ oxalic acid in the six hours of urine collection is expressed as a fraction of the total ingested dose (mg) of $[^{13}C_2]$ oxalic acid.

Parents recorded a 72-hour food diary for each child. A research dietitian performed all food analyses. Computer-based assessments of dietary calcium, sodium and protein were made using the 4.1 version of the Nutritionist IV First Databank program. Published data were used to estimate the oxalate content of food [37, 38].

Methods for molecular analysis of human liver glycolate oxidase

Genomic DNA was isolated from blood using standard methods. Control DNA samples were obtained from the University College London Hospitals Primary Hyperoxaluria Service. The coding region of the *GO* gene was ascertained by comparison of genomic (Genbank accession number AL021879) and cDNA (Genbank accession number AF244134) sequences. Primer pairs inclusive of intron-exon boundaries were designed for each of the eight exons comprising the *GO* gene. The selected oligonucleotide primer pairs for each exon, with their expected amplicon lengths are in Table 1.

The optimized annealing temperatures (T_a) for the corresponding primer pairs of exons 1 to 8 were 50, 52, 56, 52, 56, 54, 54 and 58°C, respectively. With the exception of exon 1, which required 2.0 mmol/L magnesium for amplification, 1.5 mmol/L magnesium was used for all other exons. Amplification consisted of an initial denaturation step (94°C) for two minutes, followed by 30 cycles of annealing (T_a), extension (72°C), and denaturation (94°C), each for 30 seconds, and a final extension for five minutes. A second set of primers (6.5F and 6.5R, T_a of 60°C) also was designed for concomitant amplification and sequencing of exons 6 and 7.

Mutation screening was performed using PCR-based single-strand conformational polymorphism (SSCP) analysis as previously described [39, 40]. Automated Sanger dideoxy sequencing of the products (both reverse and forward directions) was completed using an ABI-3100

Table 2. Clinical features at presentation

Patient/ Gender	Age	Symptoms	Number of stones	Medications	Stone type
1 M	4 mos	Fever, UTI	Multiple small bilateral stones, L-UVJ stone (1)	None	100% COD
2 M	24 mos	Penile & suprapubic pain, microscopic hematuria	L-kidney (1), bladder stone (1)	None	Core: 80% COM, 20% CaP Shell: 70% COM, 30% COD
3 F	10 mos	UTI, microscopic hematuria	R-kidney (3), R-ureter (2), L-kidney (3)	None	45% COM, 40% COD, 15% CaP
4 M	5 yrs 5 mos	Pain, inability to void	R-kidney (3), L-kidney (1), distal urethral stone (1)	Iron supplement	100% COM
5 M	8 mos	Gross hematuria	Multiple small bilateral parenchymal calcifications, R-proximal ureteral stone (1)	None	100% COD
6 M	4 mos	Asymptomatic, family screening	R-kidney (1)	None	100% COM

Sequencer. When appropriate, sequence changes were confirmed by restriction endonuclease digestion.

RESULTS

The clinical features of the six children at presentation are summarized in Table 2. The mean age was 19.2 months (range 4 months to 5 5/12 years). Four of the six children were younger than 12 months of age at the time of initial clinical evaluation. Patient 4 was delivered at 35 weeks of gestation and weighed 2.25 kg. He required a brief period of enteral tube feedings but not parenteral alimentation in the neonatal period. Neither hypercalciuria nor nephrocalcinosis was detected in this child. The other five children were delivered at term following uncomplicated pregnancies.

In patient 1, ultrasound examination at presentation revealed bilateral renal duplication with increased echogenicity of the medullary pyramids on the right. The latter finding resolved by the age of 19 months. No increase in medullary echogenicity was seen in the left kidney. A voiding cystourethrogram was normal in this patient. In patient 5 a transient increase in echogenicity of the renal parenchyma, with a radiologic appearance suggestive of early nephrocalcinosis, was observed on an excretory urogram at initial presentation but it resolved by the age of three years. Kidneys and urinary tracts of the remaining four children were structurally normal by ultrasound examination ($N = 2$), intravenous pyelography ($N = 1$), or computed tomography ($N = 1$).

With the exception of a very young infant in family 2, all parents and siblings were screened for hyperoxaluria and hypercalciuria. Patients 5 and 6 are siblings. There are also two unaffected children in this family. Aside from this affected sibling pair, no other first-degree relatives were found to have hyperoxaluria or are known to have urolithiasis. Hypercalciuria was present in the father of patient 1, both parents of the affected sibling pair (patients 5 and 6), and both parents of patient 4. A history of urolithiasis in more distant relatives was found in all five of the families. A family history of renal failure was not present in any family.

The laboratory features at presentation are summarized in Table 3. Urine oxalate was 1.3 ± 0.5 (1.3) mmol/1.73 m^2/24 h, mean $\pm$ SD (median), plasma oxalate 2.0 ± 0.3 (2.0) μmol/L, serum creatinine 0.5 ± 0.1 (0.5) mg/dL, and 24-hour creatinine clearance was 90 ± 19 (87) mL/min/1.73 m^2. Patient 3, with the lowest initial clearance, had left pyelocaliectasis and ureterectasis requiring pyelolithotomy when first evaluated. After relief of the obstruction, her creatinine clearance was 95 mL/min/1.73 m^2.

At presentation, urine glycolate was normal in four children and mildly elevated in two (patients 1 and 3). Urine glycerate was normal in all. Subsequent determinations were normal in all children except patient 3. At last follow-up at age 17 years, both her urine glycolate (246 μg/mg creatinine) and L-glycerate (48 μg/mg creatinine) were elevated. Urine screened for organic acids was otherwise normal in all patients, as was urine for amino acids. Hepatic AGT enzyme activity was normal in the five children who underwent biopsy. Hepatic HPR activity was normal in four children and below normal in patient 2. Hepatic GR activity was normal in the four children in whom this assay was performed. This was not measured in patient 2 due to unavailability of assay at the time of testing and insufficient amounts of tissue available for later study.

Urine calcium was normal in four patients (2.2 ± 0.56 mg/kg/24 h, median 2.2) and elevated in two (patients 1 and 5). Patient 1, who presented at the age of four months, had a calcium excretion rate of 9.5 mg/kg/24 h at initial evaluation. The hypercalciuria persisted and was 5.6 mg/kg/24 h at four years of age. His serum calcium was normal throughout. In patient 5, at the time of presentation (8 months of age), his urine calcium/creatinine ratio was 0.6 and the total serum calcium was

Table 3. Laboratory features at presentation

Patient	Urine oxalate *mmol/1.73 m²/24 h*	Urine calcium *mg/kg/24 h* [54]	Total serum calcium *mg/dL*	Serum creatinine *mg/dL*	Creatinine clearance *mL/min/1.73 m²* [55][b]	Urine glycolate *µg/mg creatinine*	Urine L-*glycerate* *µg/mg creatinine*	Hepatic AGT *µmol/h/mg protein*	Hepatic HPR *nmol/min/mg protein*	Hepatic GR
1	0.91	9.5	10.0	0.3	79[c]	116	Undetectable	29.3[f]	950	227
2	1.47	2.0	10.3	0.6	94[e]	20	9	23.0[f]	172	Not done
3	1.25	2.4	10.4	0.4	68[d]	88	Undetectable	44.2[g]	504	91
4	0.92	1.6	9.9	0.5	123[e]	12	Undetectable	37.1[g]	436	131
5	2.20	6.5	10.9	0.4	78[d]	27	Undetectable	41.2[f]	547	120
6	1.30	2.9	10.7	0.6	95[c]	18	Undetectable	Not done	Not done	Not done
Normal ranges	0.11–0.46	<4[a]	9.6–10.6	0.2–0.7	[c]67–81 [d]80–108 [e]102–150	<70	<19	[f]17.9–38.5 [g]19.1–47.9	322–1002	49–213

[a] May be higher in infants
[b] Normal values vary by age

10.9 mg/dL, with an ionized fraction of 5.5 mg/dL. Intermittent hypercalciuria and normal serum calcium were observed over time. When last evaluated, at age 14 years, his total serum calcium was 9.7 mg/dL and urine calcium was 1.2 mg/kg/24 h. PTH concentrations were normal (2.3 ± 0.3 pmol/L, median 2.4, $N = 4$), as were serum levels of calcitriol (40.4 ± 20.4 pg/mL, median 47, $N = 5$), including both hypercalciuric children. Urine citrate was 1272 ± 249 (median 1187, range 978 to 1622 mg/g creatinine). Values were normal for age in all children [41].

The study data are shown in Table 4. Dietary intakes of oxalate and calcium were 42 ± 31 (35) mg/day, and 779 ± 398 (624) mg/day, respectively. None of the children had clinical or laboratory evidence of malnutrition and stool fat determinations were normal (1.3 ± 1.7 g/day). Blood levels of pyridoxal 5′-phosphate were normal in all children. At the time of the study, urine oxalate was 1.5 ± 0.5 (1.5) mmol/1.73 m^2/24 h and iothalamate clearance was 145 ± 58 (123) mL/min/1.73 m^2. Fractional oxalate excretion was 4.2 ± 1.4 (4.0). Recovery of [$^{13}C_2$] oxalic acid in the urine after an oral load was used as a marker of enteric oxalate absorption. The total recovery during six hours following oral administration, expressed as a percent of the total ingested load of [$^{13}C_2$] oxalic acid, was 9.4 ± 3.6% (8.0%), compared to 7.6 ± 1.2% (8.0%) in the six age-matched controls ($P = 0.33$). No correlation between % [$^{13}C_2$] oxalic acid recovery and urine oxalate excretion rates was observed ($r = 0.13$).

The renal handling of phosphate as determined by $TmPO_4$/GFR (mg/dL) was 4.5 ± 1.0 (5.1) and within age-specific normal ranges in the five children that were tested. Additional parameters of renal tubular function in these five children included the urine protein/osmolality ratio 0.07 ± 0.06 (range 0.01 to 0.14), urine glucose 3.4 ± 1.8 (range 1 to 5) mg/dL and FE_{UA} 14.7 ± 6.9 (range 7 to 24) %. No abnormalities were detected.

Patients were followed for 8.7 ± 6.0 (9) years. Persistent hyperoxaluria was observed in all children during the 52 patient-years of follow-up (Fig. 2). Pyridoxine doses of 5.4 ± 2.6 (5.2) mg/kg/day had no effect on the oxalate excretion rates of any of the children. Additional treatment measures included neutral phosphates and oral fluid hydration to maintain daily urinary volumes in excess of 0.75 liter in infants, 1.0 liter in preschoolers, 2.0 liters in school age children, and 2.5 liters in adolescents. Adjunctive therapy with thiazides (patient 1) and/or citrate (patients 1 and 4) was used when indicated. Four of the patients spontaneously passed stones. Eight urologic procedures for management of stones were required in the six children. These included one open cystolithotomy, one pyelolithotomy, two ultrasonic lithotripsies (percutaneous and ureteroscopic), one laser lithotripsy (ureteroscopic), two piezoelectric lithotripsies and one ureteroscopic stone extraction. The mean time from initial presentation to stone recovery by urologic procedure

Table 4. Study data

Patient	Age @ study *years*	Dietary oxalate *mg/day*	Dietary calcium *mg/day*	Dietary protein *g/kg/day*	Urine oxalate *mg/day*	Iothalamate clearance *mL/min/1.73 m²*	Plasma oxalate *μmol/L*	FE_{Ox}	Enteric oxalate absorption *%*
1	3.3	8[a]	451	3.6	48.4	248	3.1	4.9	4.9
2	5.3	51	426	2.3	57.2	104	2.3	4.2	14.3
3	15.6	23	1289	1.2	74.8	94	1.3	6.4	7.8
4	8.4	33	529	2.2	49.3	178	1.9	3.8	8.1
5	12.2	38	719	1.0	137.3	129	0.9	2.1	13.3
6	9.9	97	1263	2.8	135.5	116	2.0	3.8	7.9

[a] Patient 1 is on an oxalate restricted diet

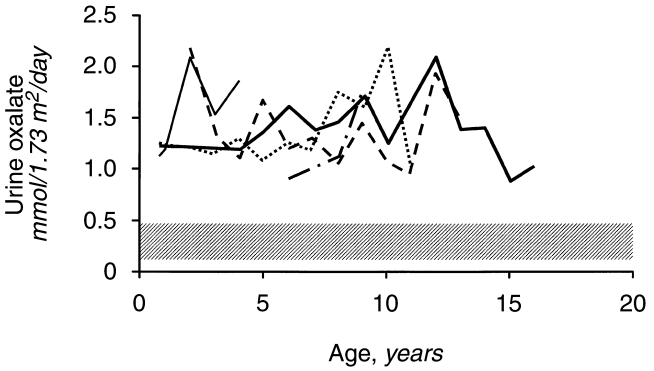

Fig. 2. Urine oxalate (mmol/1.73 m²/24 h) in children with hyperoxaluria and urolithiasis. Moderate to marked hyperoxaluria persisted throughout follow-up. The normal range is indicated by the shaded area.

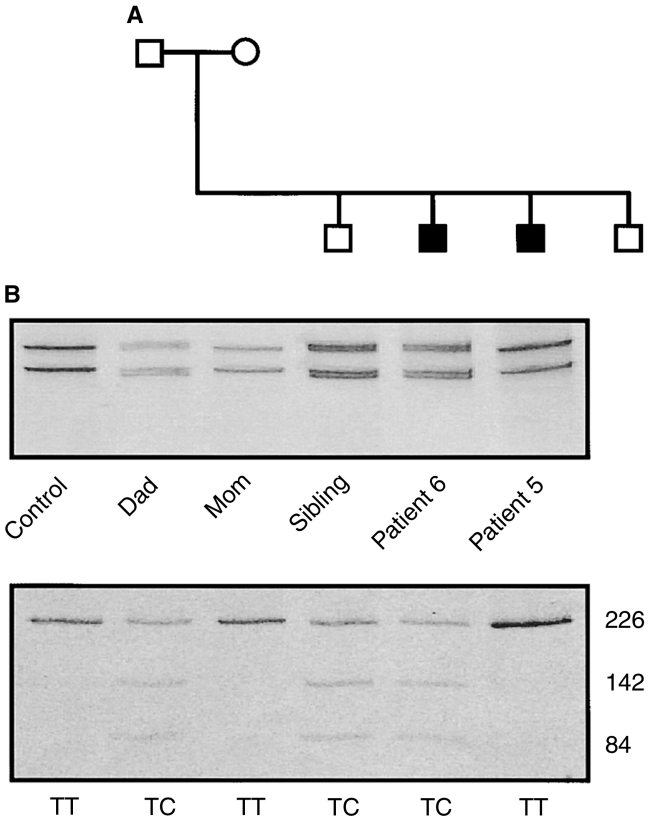

Fig. 3. (*A*) Family 5 pedigree. Family members with hyperoxaluria and urolithiasis are indicated by (■). There were no family members with hyperoxaluria alone or urolithiasis alone. (*B*) SSCP analysis (upper panel) and *Hae III* endonuclease digestion (lower panel) of exon 5 GO. Letters indicate the genotype at nucleotide 754. The PCR fragment size is 226 bp. The T754C change introduces a *Hae III* restriction site at position 84. Numbers to the right indicate the lengths (bp) of the digested products.

was 10.3 months (range 0 to 29, median 5.5). Age at last follow-up was 10.8 ± 5.2 (11) years, with a measured creatinine clearance of 107 ± 14.8 (112) cc/min/1.73 m².

Genetic analysis of the glycolate oxidase gene

Preliminary work with glycolate oxidase (GO) as the candidate gene for a third type of inherited hyperoxaluria excluded deficiency of this enzyme as the cause of hyperoxaluria in family 5. Analysis of family pedigrees was consistent with an autosomal recessive pattern of inheritance. Accordingly, we anticipated the affected sibship (patients 5 and 6) to share the same two mutant GO alleles, one inherited from each parent, both assumed heterozygous carriers for the causative mutation. The SSCP patterns and sequencing results of exon 5 in this family, confirmed by *Hae III* endonuclease digestion, however, indicated the presence of different alleles in the two affected brothers (Fig. 3). Patient 6 showed heterozygosity for a synonymous T754C change (codon 252 TTG > CTG) while patient 5 was homozygous for the wild-type sequence (TT). Thus, the pattern of inheritance of this T754C sequence variant effectively excluded *GO* as the candidate gene in this family.

Comprehensive screening by direct sequencing of all GO exons and exon/intron boundaries was carried out in the remaining four patients (patients 1 to 4). Amplification and sequencing of exon 4 revealed an IVS3 – 49 C>G change. This sequence change was homozygous in patients 1 and 2 and heterozygous in patient 4. Patient 3 was homozygous for the wild-type sequence (CC genotype), as was a control patient from University College London. No other sequence variation was identified by di-

rect sequencing of the remaining exons and exon/intron boundaries of the *GO* gene in any patient.

DISCUSSION

The distinctive clinical features of the children in this report are the young age at stone development, with multiple calculi during infancy in four of six children, and the persistent, moderate to marked hyperoxaluria of no identifiable primary or secondary cause. The finding of two affected siblings in one family suggests a genetic basis.

We excluded both known types of inherited hyperoxalurias, PHI by normal levels of AGT, and PHII by normal levels of GR and/or HPR and urinary L-glycerate. Patients with PHII typically have markedly elevated urinary L-glycerate [42], however, there is a recent report of normal L-glycerate in two siblings with PHII [43]. The possibility of type II primary hyperoxaluria remains in patient 2 from our cohort, who had a mild reduction in hepatic HPR activity and normal urine L-glycerate. The unavailability of sufficient hepatic tissue and of the GR assay at the time of his screening precluded unequivocal exclusion of PH2.

Mutation of glycolate oxidase could potentially contribute to hyperoxaluria either by increasing the affinity of the enzyme for glyoxylate and thereby increasing intraperoxisomal oxalate production or, in deficiency states, by conversion of its substrate glycolate to oxalate by as yet undefined pathways. Mutation screening of the intron/exon boundaries and entire coding region of the *GO* gene did not reveal any disease-associated sequence changes in any of the five families. The two detected sequence variants (T754C and IVS3-49 C>G) are likely polymorphisms. Nonetheless, many of the pathways participating in hepatic metabolism of glyoxylate, and hence endogenous oxalate production, remain enigmatic [44] and the possibility of hepatic candidate enzymes other than GO accounting for the hyperoxaluria in this subset of children remains to be explored.

Likewise, the net summation interaction of the known various hepatic enzymes culminating in endogenous oxalate synthesis (AGT, GR/HPR, GO, LDH) has not been evaluated. Inborn errors of metabolism often show complex phenotypes and partial defects of different enzymes in a pathway may account for elusive clinical and biochemical findings, a concept termed 'synergistic heterozygosity' [45]. This is particularly exemplified by patient 2 whose AGT level was low normal and HPR activity was reduced. The combined effect of known molecular variation, such as the C154T polymorphism of AGT [46], and of other genes coding for enzymes in the glyoxylate pathway on cumulative hepatic oxalate production awaits further investigation. For instance, 'down-regulation' of any one enzyme resulting from a given sequence change, without documented deficiency, may affect the net flux of metabolites through the pathway, and is yet another potential mechanism for the hyperoxaluria in these children.

Organic acids and amino acids are involved in the metabolic pathways leading to formation of oxalate. An abnormality detected on urine screening for organic acids or amino acids would direct attention to the relevant component of the metabolic pathway as a potential cause of the hyperoxaluria. However, aside from the hyperoxaluria, organic acid and amino acid screens were normal in our patients.

We excluded known secondary causes of hyperoxaluria. Plasma pyridoxal 5′-phosphate concentrations were normal in all of the patients. Dietary oxalate intake was not excessive in any child and neither gastrointestinal disease nor malabsorption was present. We further evaluated enteric oxalate absorption as a potential source of the hyperoxaluria by measuring [$^{13}C_2$] oxalate in the urine after ingestion of an oral load. There was no difference ($P = 0.33$) in % recovery of [$^{13}C_2$] oxalate between patients and age-matched controls.

A potential role for the gastrointestinal tract in the degradation of dietary oxalate has emerged recently. Colonization by several species of oxalate-degrading bacteria, including *Oxalobacter formigenes* [47] and lactic acid bacteria (*L. acidophilus, S. thermophilus*) [9], has been shown to alter the oxalate excretion rates of some hyperoxaluric stone formers [9]. A decrease in the amount of oxalate available for absorption within the colonic lumen as a result of the oxalate degrading ability of these bacteria is postulated to account for the observed reduction. Effects of colonic bacteria on oxalate degradation may have been difficult to detect under the conditions of our oxalate absorption study. However, urine oxalate excretion is often normal in the absence of oxalate degrading bacteria in the intestinal tract [47]. The absence of oxalate degrading bacteria seems an unlikely mechanism in our cohort of patients, as their urine oxalate excretion rates were greater than the total dietary oxalate intake by a mean factor of 2.0 and these rates were sustained in this range over many years.

Hypercalciuria was a second risk factor for stone formation detected in two of the six children (patients 1 and 5). While we cannot conclusively exclude a common renal tubulopathy accounting for the marked hyperoxaluria and hypercalciuria in the two hypercalciuric children, the lack of linkage of the two traits in either family (families 1 and 5), the intermittent nature of the hypercalciuria in patient 5, and its absence despite hyperoxaluria in this patient's sibling, patient 6, suggests an independent cause for the abnormal calcium excretion.

Heightened renal tubular secretion of oxalate as the sole explanation for the hyperoxaluria in these children also does not seem likely given the degree of hyperoxaluria and the normal plasma oxalate concentrations ob-

served. Fractional excretion of oxalate in these children was similar to that observed in patients with primary hyperoxaluria [22].

Renal oxalate synthesis is suggested by recent data [48]. AGT activity was detected in cultured renal proximal tubular (LLC-PK1) cell lines and in renal cortex from harvested human kidney tissue, but not in distal tubular (MDCK) cell lines or in sections of medullary or papillary renal tissue. GO activity was not identified in either the harvested kidney tissue or the cultured renal cell lines. However, there were detectable levels of oxalate and glycolate in all portions of harvested renal tissue and measurable oxalate synthesis in the cultured MDCK cells, suggesting that other glyoxylate metabolizing enzymes may contribute to oxalate production by the kidney [48].

Moreover, two different isoforms of alanine:glyoxylate aminotransferase, AGT1 and AGT2, have been described previously in mammals [49], the latter form being present predominantly in rodents (rat liver and kidney) [50, 51]. Although kinetically and structurally distinct from human liver AGT (AGT1), AGT2 has a conserved PLP binding site, shows homology to other aminotransferases [50], and has the ability to catalyze the same reaction (that is, the transamination of glyoxylate to glycine using alanine as the amino donor) [49]. The functional properties of this enzyme in kidney tissue have not been fully elucidated and while previously deemed absent in humans [49, 52], the corresponding human messenger RNA sequence was recently deposited in GenBank (accession number AJ298293/XM040434). Hence, a potential role for kidney tissue in endogenous oxalate synthesis, and as a cause of hyperoxaluria in these children, cannot be entirely excluded based on the information available to date.

Four other children with hyperoxaluria of no identifiable cause have been described in the literature. Van Acker et al reported hyperoxaluria and hyperglycolic aciduria with normal activity and localization of AGT in two unrelated patients [53]. Hepatic levels of GR/HPR were not measured and a diagnosis of type II primary hyperoxaluria was excluded by absence of L-glycerate on urinary organic acid screening. Neither patient had urolithiasis or nephrocalcinosis. More recently, two children with moderate hyperoxaluria, early onset calcium oxalate urolithiasis, normal urine glycolate and L-glycerate, and normal hepatic levels of AGT and GR/HPR were identified by Neuhaus et al [6].

To our knowledge, this is the first systematic evaluation to ascertain the underlying cause of hyperoxaluria in a group of such children. The marked and sustained hyperoxaluria, and involvement of two siblings in one family, is most consistent with a metabolic, perhaps heritable, origin. Metabolic overproduction of oxalate might occur by an as yet unrecognized hepatic enzyme pathway or, alternatively, in renal tubular cells. A single mechanism may not account for the findings in all patients reported here. Confirmation of the source of hyperoxaluria in these children awaits further study.

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